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Characterizing SHP2 as a Novel Therapeutic Target in Breast Cancer


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roles of different acidic residues in the binding of the Src homology 2-containing *ã~\æ↔^Á\]ã~b↔^æÁ
phosphatase (SHP2) to its substrates. A peptide derived from this substrate }ábÁbå~}^Á\~ÁâæÁáÁbæ→æ´\↔{æ
inhibitor of SHP2. SHP1 was used as a control, and the peptideÁä↔äÁ^~\Áá´\Á~^Á\å↔bÁæˇ˘]↑æÈ
Molecular modeling revealed key binding determinants at the á\~↑↔´Áæ{æ→Á*ãæbæ^\Á↔^ÁUÕŞGÁ\åá\
could not be recapitulated in SHP1. These modelingÁ~âbæã{á\↔~^bÁ}æãæÁ\æb\æäÁ|b↔^&Á↑\á\↔~^Á~àÁ\åæÁÁ
peptide. Indeed, when the peptide was mutatedÁ\~Áábâ~→↔båÁ\åæbæÁ^\æãã´\↔~^bÊÁ^å↔å↔â↔\~ã]Á´á*á´↔\]Á
was abolished entirely. Systematic ↑|\á\↔~^Á~àÁ\åæÁ*æ*\↔äæÁ^Áb↔→↔´~Áà|ã\åæãÁä↑~^b\ãá\æäÁ\åá\ÁÁ
the substrate needed to bind both ~àÁ\åæÁ*~b↔\↔{æ→]Ë´åáã&æäÁÁ↑↔^Á´↔äÁb↔äæ´åá↔b´\á´æ^\
to the active site to facilitate the å↔&åæb\Á&↔^ä↔^&Áb\áâ↔~→→\]ÈÁ́R|\á\↔~^Á~àÁæ^]Á~àÁåæ
acidic amino acids next to the *å~b*å~\]ã~b↔^æÁ~^Á\åæÁb|âb\ãá\æÁ̃~ä|´æäÁ↔&↔↔à↔´á^Á̃ääæàá´\bÁ↔↔^
binding as predicted by ↑~→æ´|→áãÁ̃ä~´↔↔^&ÈÁÚúåæbæÁä↔^äÁ↔^&bÁãæÁ~}á↔^↔&Á*áã→áæäÁ^^\~Áb\|ä]Á~àÁåæ
peptide-basedÈ

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**Introduction**

The Src homology 2-containing protein tyrosine phosphatase (SHP2) is a positive regulator of cellular signaling and promotes breast cancer tumorigenesis.  Because of this, it was hypothesized that SHP2 may be a useful therapeutic target in disease, since it acts as an integrator of numerous signaling pathways that are known to be dysregulated in cancer such as HER2.  Previous cell biology work has demonstrated that SHP2 is required for maintenance of transformation.  It has not been conclusively demonstrated that SHP2 would serve as an attractive therapeutic target.  In addition, SHP2 inhibitors designed so far have failed to demonstrate selectivity over closely-related homologues such as SHP1 (1,2).  This work was designed to first determine how SHP2 selectively binds its target substrates and then to apply this knowledge in the first-in-class demonstration of SHP2's viability as a therapeutic target in breast cancer.

**Body**
Over the course of the second year of study, research efforts have been focused on the statement of work tasks 3 and 4.  They include the following overarching goals:

**Task 3- Synthesize and characterize anti-SHP2 peptides derived from the Y1023-HER2 in vitro (Months 13-20)**

**Task 4- Molecular modeling of HER2 peptides (Months 13-20)**

Both of these tasks serve a synergistic purpose.  *In vitro* characterization of the substrate-derived peptide was used to confirm the predictions based on molecular modeling.  Therefore, the results of this line of work will be discussed together.

*The substrate-based peptide is a SHP2 inhibitor that does not target SHP1*
        Tyrosine 1023 of HER2 becomes phosphorylated in response to different stimuli, including EGF.  SHP2 is known to act upon this site, dephosphorylating it.  Dephosphorylation prevents the binding of the signaling inhibitor RasGAP, resulting in enhanced signaling (3). Since this is the only site on HER2 known to be engaged by SHP2, it was hypothesized that a peptide derived from the substrate sequence could act as a selective inhibitor.  Thus, the peptide sequence DADEpYL was synthesized (pY representing phosphotyrosine) and tested against SHP2 and SHP1 activity.  The Cheng-Prusoff equation was used to convert the $IC_{50}$ into the inhibitory constant, $K_i$.  The results are reported in Table 1.  This peptide was found to have a Ki against SHP2 of 11.92 micromolar, whereas in SHP1 the inhibitory constant was found to be 630 micromolar.  This large selectivity for SHP2 is unprecedented in the design of small molecule inhibitors.  Therefore, the substrate-derived peptide was shown to be significantly more selective for SHP2 compared to its close homolog, SHP1.

*Structural modeling predicts key interactions in the selective binding of the substrate-based peptide to SHP2.*
        A preliminary analysis of the primary sequence of the two enzymes revealed some differences in the region surrounding the active site (Figure 1A).  Specifically, lysine 364 (K364) was found to be substituted with an arginine in SHP1, and arginine 362 (R362) of SHP2 was replaced by a lysine in SHP1.  These residues have been previously suggested to play roles in selective binding of inhibitors(4).  To understand how these differences may translate into conformational alteration of the active site, the crystal structures of SHP2 (2SHP.pdb) and SHP1 (1GWZ.pdb) were overlaid based on the catalytic region (Figure 1B).  K364 in SHP2 was shown to be placed in the same location as an arginine in SHP1.  These residues were conserved in terms of sidechain charge, but they differed significantly in terms of structure.
        Since we found that the substrate-based peptide displayed selectively for inhibition of SHP2 activity over, we wished to identify the intermolecular interactions that might be critical for binding to both enzymes.  Since no crystal structure of SHP2 bound to this phosphopeptide is available, we attempted to elucidate interactions with molecular docking (5). The peptide was docked into the SH2 active site of 2SHP.pdb (with SH2 domains stripped from the crystal) using the genetic algorithm.  At least 2000 runs were performed, and the most favorable conformation was selected (Figure 2).  We ensured that the phosphotyrosine was situated inside the active site near the catalytic cysteine, an essential characteristic of binding in the PTP mechanism(6). Electrostatic interactions were computed in Autodock Tools.  Strong ionic contacts were found between arginines and lysines to the acidic residues of the substrate peptide (Table 2), with the most prominent being the -2 position aspartate residue to K364 (-6.737 kcal/mol).  Other strong predicted interactions included two contacts with the -4 position aspartate of the peptide to arginine 362 (-2.827 and -3.653 kcal/mol).  The -2 aspartate also interacted with lysine 366 (-0.113 kcal/mol).  Finally, the -1 glutamate residue of the peptide was predicted to hydrogen bond with Y279 of SHP2 (-2.903 kcal/mol).  The total intermolecular interaction energy was found to be -14.3 kcal/mol with -8.50 kcal/mol originating from Van der Waals interactions and

hydrogen bonds and -5.80 kcal/mol provided by electrostatic interactions.  Overall, the predicted Ki of the peptide was 9.72uM, which aligned reasonably well with the kinetics data we obtained.

Differences in selectivity for the peptide inhibitor were predicted by docking the same substrate-derived peptide into the SHP1 active site (Figure 3).  Out of more than 2000 runs, the best docked structure predicted a Ki of 641 uM, a rather dramatic loss of predicted inhibition compared to SHP2.  Analysis of the structure revealed many electrostatic contacts between the peptide and the active site (Table 2).  Arginine 358, which is analogous to lysine 364 of SHP2, was predicted to form an ionic bond with the -2 position aspartate of the peptide (-4.518 kcal/mol)  Another electrostatic interaction was found between histidine 420 and the -1 glutamate of the peptide (-3.67 kcal/mol).  The geometry of the predicted interactions was influenced by steric clashes.  The orientation and size of arginine 358 appeared to create a pocket that required deformation of the peptide and atom contacts with the active site, resulting in a lowered overall binding energy.

In order to further corroborate the docking calculations of the wildtype peptide, docking was used to perform an *in silico* alanine scan.  This was done by mutating the peptide's acidic amino acids into alanine and predicting the new binding conformation and affinity of the molecule.  In this way, three new peptides were assessed, mutating at the -4, -2, and -1 positions.  The results of this experiment are reported in Table 3.

The -1 mutant peptide, DADApYL, demonstrated a significantly poorer predicted inhibition constant when compared to the wildtype peptide.  The constant predicted for this peptide was found to be 493.5 µM.  The predicted binding conformation of this peptide showed a tendency to form polar contacts with lysine 364 (Fig. 4).  The peptide AADE predicted an even more pronounced loss of inhibition by the peptide, 608.21 µM.  Both lysine 364 and 366 were targeted for interaction by the peptide (Fig. 5).  Finally, the DAAEpYL peptide was docked, yielding a predicted inhibition constant of 611.38 µM.  The most prominent sidechain interaction was found between lysine 364 and the -1 glutamate residue of the peptide (Fig. 6).  This predicted inhibition is in reasonable agreement with the experimental results reported in Table 1 for this peptide.

The *in silico* alanine scan demonstrated two major findings about the binding of substrate peptide to SHP2.  First, each of the acidic amino acids adjacent to the phosphotyrosine appears to play a critical role, since mutation of any of these engenders a dramatic loss of predicted binding capacity.  Second, the peptides appear to show a preference for binding the lysine 364 residue to the exclusion of arginine 362 that was suggested to also play a deciding role in binding of the wildtype peptide.  Since arginine 362 was not targeted, it would appear that binding to this residue is a key constituent of strong binding that leads to the low micromolar affinity of the wildtype peptide.  Both the lysine 364 and arginine 362 form strong interactions with the peptide.  This is particularly compelling, because, as noted in Figure 1, these two residues are swapped in SHP1, and the wildtype peptide cannot dock and form the same interactions as strongly with the new residues.

When combined, these data present strong evidence that the driving force of SHP2 selectivity is the interaction of molecules with both the lysine 364 and arginine 362 residues adjacent to the active site.  This information will prove valuable in the future design of inhibitors in this field, since to date no drug has been designed that specifically targets the lysine 364 and arginine 362.

*The substrate-based peptide is an inhibitor of SHP2-mediated cell signaling in cancer cells*

The *in vitro and in silico* data of the peptide were compelling evidence for the substrate-derived peptide's ability to inhibit SHP2 activity in a selective manner.  Next, it was determined what kind of effect this peptide might have on cells.  As laid out in task 6 (Test the efficacy of peptide inhibitors against SHP2-mediated cell signaling and transformation), I tested the hypothesis that the peptide could act to inhibit SHP2 in cells, conferring an effect on signaling similar to that seen when SHP2 expression is inhibited using short-hairpin RNA.  Specifically, suppression of SHP2 inhibits the ability for EGF to stimulate the mitogen-activated protein kinase (MAPK) pathway, which is read as a drop in phosphorylated ERK protein(3).

First, the problem of cell permeability needed to be overcome.  The phosphobenzene moiety of the peptide confers significant negative charge to the peptide, making it unlikely to enter the cell spontaneously.  To solve this problem, the peptide sequence was altered to include the cell-penetrating sequence from the HIV TAT1 protein, which has been shown to facilitate delivery of different types of cargo into cells (7).  The peptide was also tagged with fluorescein isothiocyanate (FITC) to determine if it was being successfully internalized into cells.  BT474 has been tested so far with this peptide.  These HER2-positive cancer cells are ideal to model EGF response and dependence on SHP2.  First, they were grown to confluence.  Once they reached this stage, the cells were serum-starved overnight.  The following morning, the peptide was incubated with the cells for one hour before EGF was added to a concentration of 100 ng/mL.  The FITC channel of an Olympus X78 microscope was used to verify internalization of the FITC tag (Figure 7).  It was noted that the phosphorylated form of the peptide was able to localize to the membrane in response to EGF, which suggests that it may be acting upon SHP2 in this region.  SHP2 is recruited to the membrane independently of HER2 through the Grb2-associated protein 2, an adapter molecule that is recruited to the membrane following EGF stimulation.  The non-phosphorylated peptide did not show this same localization, but it was significantly internalized thanks to the TAT1 tag.

Under the same conditions, EGF-mediated activation of the MAPK signaling pathway was tested in the presence of the phosphorylated and non-phosphorylated peptides (Figure 8).  The non-phosphorylated peptide showed no change in the signaling intensity or duration, while the phosphorylated form strongly inhibited both the initial spike and the duration of phosphorylated ERK signal.  These results strongly suggest that the substrate-derived peptide inhibitor can suppress SHP2 activity in cells in addition to *in vitro* experiments.

In the coming months, this peptide will be tested in other cell lines and against other phenotypes related to cancer.  These include migration and anchorage-independent growth.

**Key Research Accomplishments**

- A peptide derived from the known SHP2 substrate HER2 is a selective inhibitor of enzymatic activity *in vitro*.
- Association with positively charged amino acids lysine 364 and arginine 362 is critical for binding SHP2.
- The same binding interactions for SHP2 cannot be recapitulated in SHP1 despite similarities in sequence.  The structure of the active site of SHP1 does not allow for the same interactions to occur.
- The substrate-derived peptide, once fused to a cell-penetrating sequence, can act as an inhibitor of SHP2-mediated signaling in HER2-positive breast cancer cells.

**Reportable Outcomes**
- Poster presentation at the 2012 WVU Van Liere Research Day
- Imminent publication of a paper in Molecular Cancer Research regarding the role of SHP2 in metastasis and cell migration (final revision), helping to further characterize SHP2 as a therapeutic target in breast cancer
- Final stages of paper submission to the Journal of Biological Chemistry regarding the determination of SHP2 selectivity


**Conclusions**

The work gathered into this report represents a significant milestone in the characterization of SHP2.  To date, many labs have attempted to design or screen for inhibitors against this enzyme with little success (2,4,8-10).  This work has yielded some very interesting observations regarding what can make for a selective inhibitor, but no group has yet reached satisfactory levels.  What the results presented here show is that selectivity can be achieved for SHP2 over SHP1 (the most challenging enzyme pair to inhibit selectively due to sequence homology), and, more importantly, it is the association of negatively-charged functional groups with the positively-charged amino acids of SHP2 adjacent to the active site that mediate the selectivity.  Specifically, interactions with both arginine 362 and lysine 364 near the active site appear to be crucial for binding to SHP2, since mutations in the peptide that abolish one of these interactions remove the inhibitory capacity.  These same interactions cannot be formed by the peptide in SHP1 as predicted by molecular docking, suggesting a strategy for selective inhibition by a peptidomimetic.

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**Supporting Data**

Table 1.  Inhibition of SHP1 and SHP2 by substrate-derived peptides.

| Peptide | Predicted Ki (μM) | Experimental Ki (μM) |
|---|---|---|
| DADEpYL | | |
| SHP1 | 641 | 630.0 |
| SHP2 | 9.72 | 11.97 |
| | | |
| DAAEpYL | | |
| SHP2 | 611.38 | 856.8 |

Table 2.  Predicted electrostatic energies of interactions between the peptide and SHP1/SHP2[a]

| Peptide to SHP2 | Energy | Peptide to SHP1 | Energy |
|---|---|---|---|
| -1 Glu to Y279 | -2.903 | -1 Glu to H420 | -3.67 |
| -2 Asp toK364 | -6.737 | -1 Asp to K356 | -0.301 |
| -2 Asp to K364 | -0.113 | -2 Asp to R359 | -4.518 |
| -4 Asp to R362 | -2.827 | -2 Asp to R359 | -0.26 |
| -4 Asp to R362 | -3.653 | -4 Asp to K356 | -0.007 |

[a] Energy in kcal/mol

Table 3.  *In silico* alanine scan of the peptide, bound to SHP2

| Peptide | Predicted Ki (μM) |
|---|---|
| DADApYL | 493.5 |
| DAAEpYL | 611.38 |
| AADEpYL | 608.21 |

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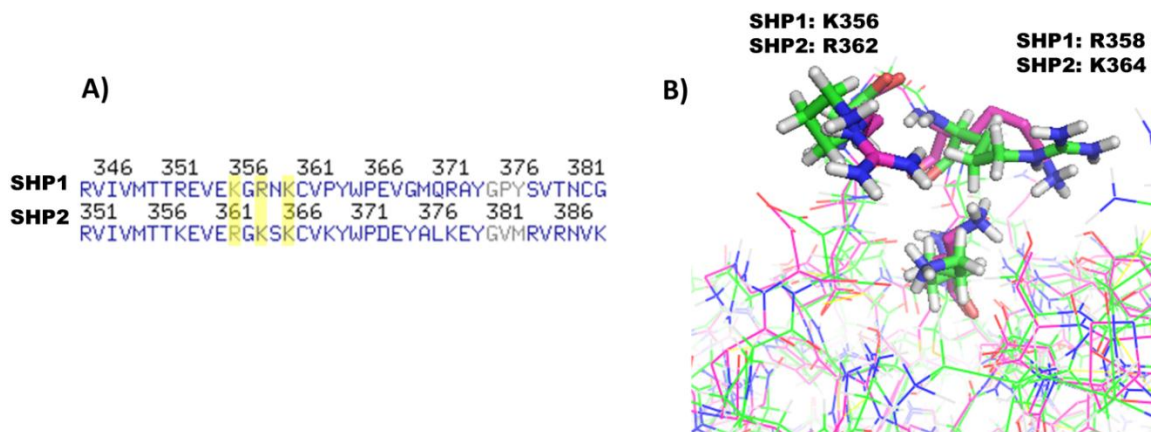
Figure 2. Comparison of the SHP1/SHP2 active sites
a) Overlay of the primary sequence of the non-conserved active site regions between SHP1 and SHP2
b) Overlay of the SHP1 (1GWZ.pdb) and SHP2 (2SHP.pdb) active sites. SHP1 backbone is colored in green, and SHP2 is colored pink. The highlighted residues from A are presented as licorice models, and the conserved active site arginine and cysteine residues are highlighted for reference.
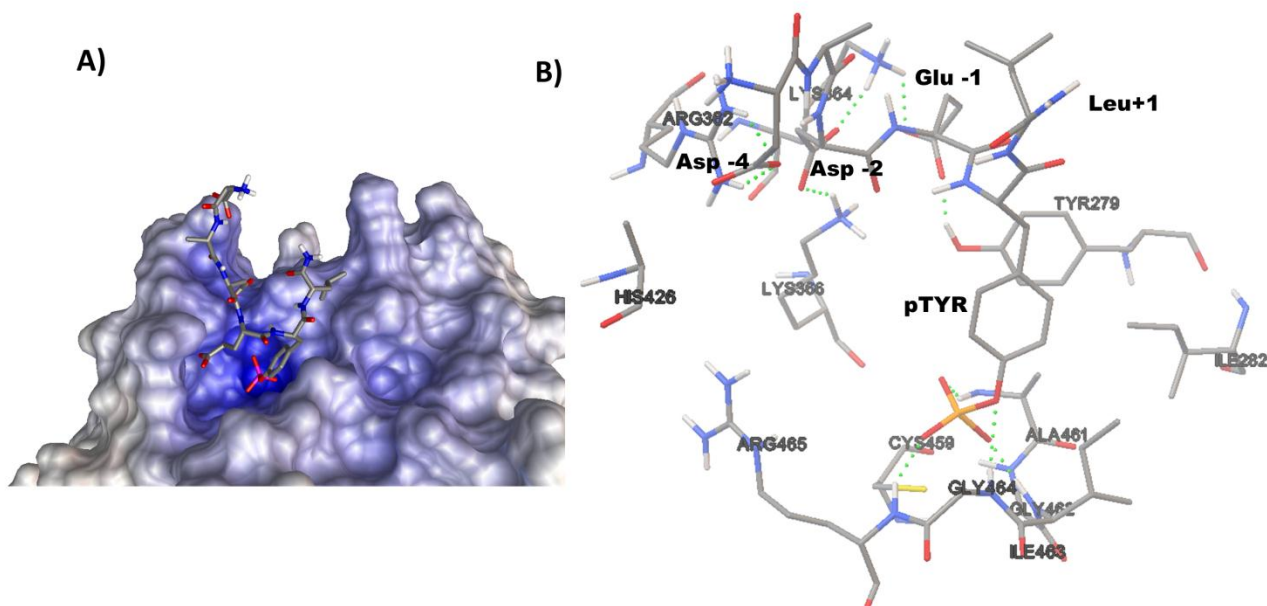


Figure 1. Computational docking of the substrate-derived peptide into the SHP2 active site
a) EGFR Y992-derived peptide was docked into the SHP2 active site using Autodock4, and the binding mode is presented with SHP2 residues represented using the CPK model. b) Interactions between the peptide and the SHP2 active sites. Peptide residues are labeled based on their sequence position relative to the phosphotyrosine. Polar contacts are indicated by green dots.
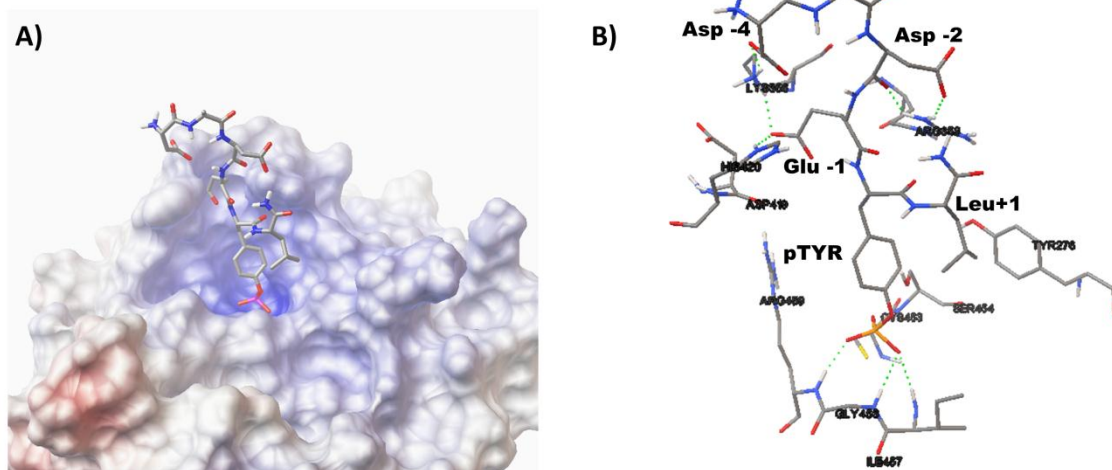
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Figure 3. Computational docking of the substrate-derived peptide into the SHP1 active site
a) Substrate-derived peptide was docked into the SHP1 active site using Autodock4, and the
binding mode is presented with SHP1 residues represented using the CPK model.  b)
Interactions between the peptide and the SHP1 active sites.  Peptide residues are labeled
based on their sequence position relative to the phosphotyrosine.  Polar contacts are indicated
by green dots.
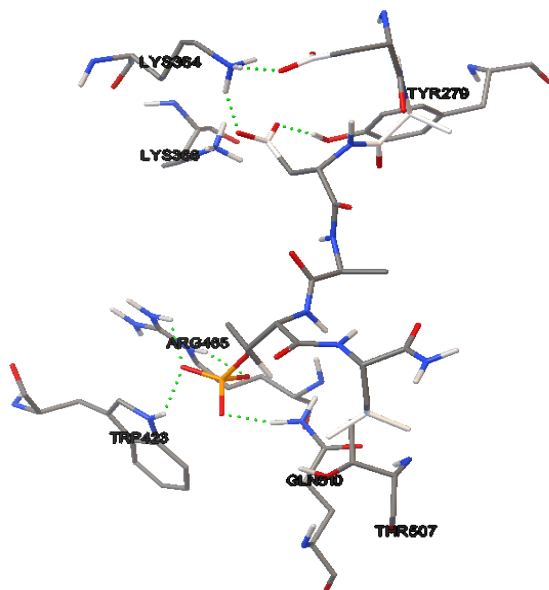


Figure 4.  Predicted binding conformation of the DADApYL peptide to SHP2.  Predicted
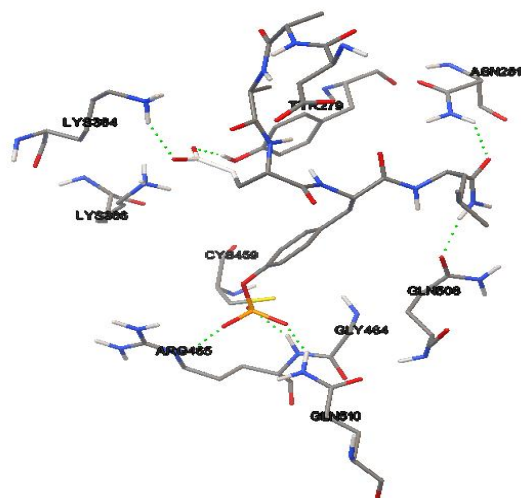hydrogen bonds are represented by green spheres.

Figure 5.  Predicted binding conformation of the DAAEpYL peptide to SHP2.  Predicted hydrogen bonds are represented by green spheres.
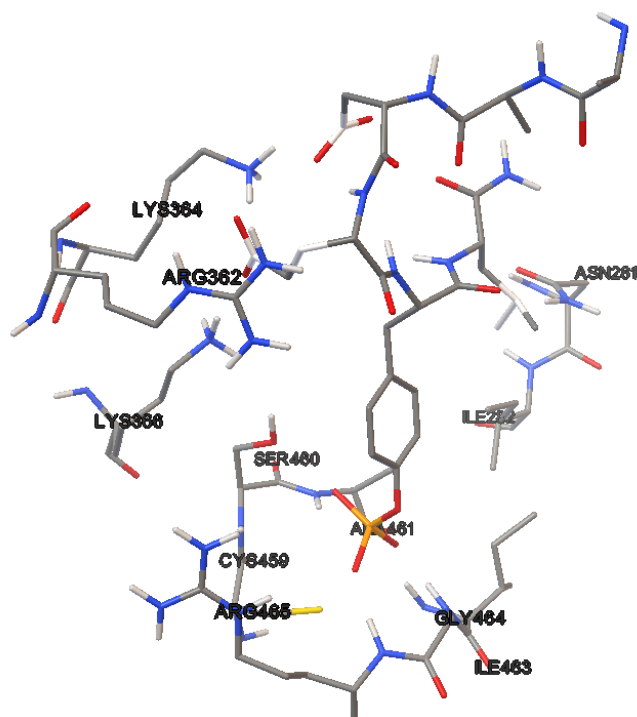


Figure 6.  Predicted binding conformation of the AADEpYL peptide to SHP2.  Predicted hydrogen bonds are represented by green spheres.

-EGF    +EGF        -EGF    +EGF
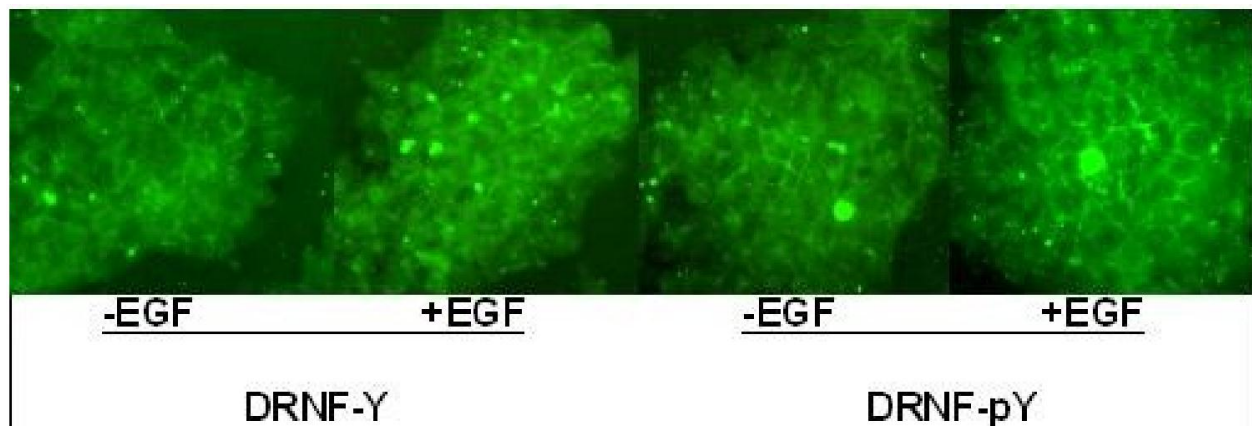
DRNF-Y              DRNF-pY

Figure 7. Successful internalization and pY-dependent membrane localization of the FITC-tagged substrate-derived peptide. BT474 cells were grown to confluence before serum-starving overnight. Then, EGF (100 ng/mL) was added in the presence of either phosphorylated peptide or non-phosphorylated peptide. Penetration into the cell was facilitated by incorporating the cell-penetrating peptide sequence from HIV-TAT1.



pERK1/2

pAkt

β-Actin

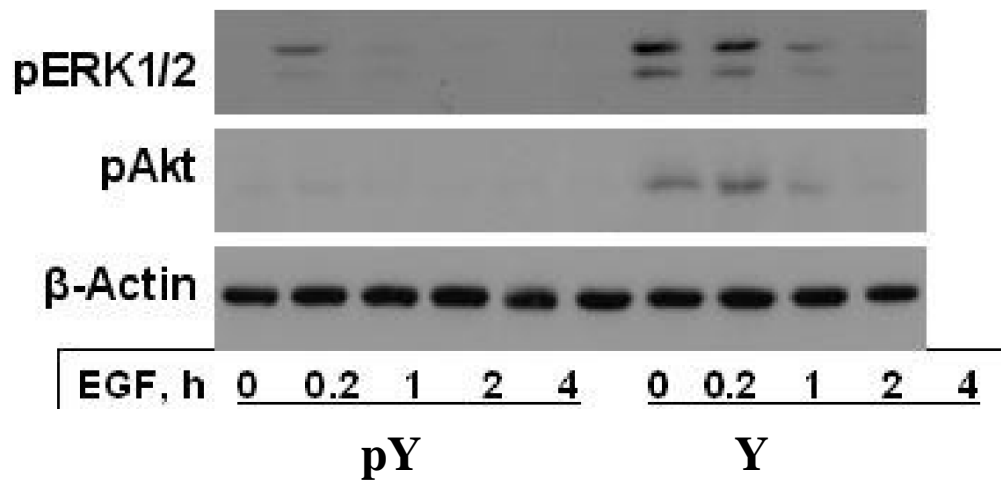EGF, h   0   0.2   1   2   4        0   0.2   1   2   4

pY                      Y

Figure 8. A phosphorylated substrate-based peptide acts as an inhibitor of signaling *in vitro*. BT474 cells were grown to confluence before serum-starving overnight. Then, EGF (100 ng/mL) was added for the indicated times in the presence of either phosphorylated peptide or non-phosphorylated peptide. Penetration into the cell was facilitated by incorporating the cell-penetrating peptide sequence from HIV-TAT1.

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